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Dated: October 25, 2010 Signature: /Jeanne M. Brashear/56,301

Docket No.: 28113/39467A
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Alitalo et al.)	Confirmation No.: 2853
)	
Serial No.: 10/567,630)	Art Unit: 1634
)	
Filed: May 30, 2006)	Examiner: Stephen T. Kapushoc
)	
For: Materials and Methods for)	
Colorectal Cancer Screening,)	
Diagnosis and Therapy)	

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. KARI ALITALO

I, Dr. Kari Alitalo, do hereby declare and state as follows:

1. I am a co-inventor of the invention claimed in the above-referenced patent application (hereinafter, "the patent application"). I currently hold the position of Research Professor of the Finnish Academy of Sciences and am the lead scientist in a laboratory in Finland staffed with post-doctoral, graduate student and other researchers. My laboratory also collaborates to varying degrees with other scientists from time to time. I make this declaration to provide to the PTO scientific data that may be pertinent to issues raised during examination of the application.

2. The invention described in the patent application is directed, in part, to a methods of screening for colon cancer in a human by measuring Prox-1 in a colon tissue sample, wherein elevated Prox-1 detected in the sample is indicative of colon cancer in the human. Exemplary claims defining the invention are appended hereto as Exhibit A.

3. I understand that the application currently stands rejected, in part because the Examiner has questioned whether the invention works as described with respect to the correlation between elevated Prox-1 protein in a sample and colon cancer. The application teaches that Prox-1 is overexpressed in precancerous and colon cancer cells and that the detection of Prox-1 polynucleotides and polypeptides is useful for diagnostic purposes. Further,

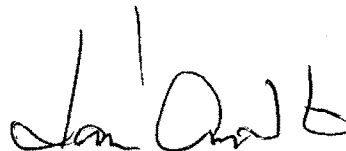
Example 2 of the application demonstrates that elevated Prox-1 protein expression was observed in 9 of 11 human colorectal adenomas samples and also in 6 of 9 carcinomas compared to adjacent normal mucosa. See, for example, pages 60-61 and Figures 2A-2I of the application-as-filed. Thus, the application-as-filed demonstrates that increased Prox-1 protein expression is observed in colon cancer compared to normal tissue.

4. Subsequent to the filing of the patent application, I have been involved in further research which confirms that colon cancer tumor samples express elevated levels of Prox-1 protein compared to healthy colon tissue samples.

5. Part of our research involved measuring (immunohistochemically) Prox-1 protein expressed in colon tissue from colorectal cancer patients and in colon tissue from healthy control subjects. A copy of a draft manuscript is attached hereto as Exhibit B which describes in detail the work that we performed and results we obtained, and which is incorporated by reference into this declaration. As described in the manuscript, 91% of the tumor samples that were tested expressed Prox-1 (471/517), whereas normal intestinal epithelium was negative for Prox-1, with the exception of a few crypt and neuroendocrine cells. The results from the experiments described in the attached manuscript are consistent with the teaching in the application-as-filed that elevated Prox-1 expression compared to healthy tissue is observed in tissue samples from colon cancer subjects. Moreover, as explained in the manuscript, within the tumor samples tested, higher Prox-1 expression correlated with unfavorable patient outcome (See, e.g., p. 8).

6. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date October 19, 2010



Dr. Kari Alitalo

Exhibit A

1. (Currently amended) A method of screening colon tissue for colon cancer, said method comprising:

measuring (prospero homeobox protein 1) Prox-1 expression ~~or activity~~ in a biological sample that comprises colon tissue from a ~~mammalian~~ human subject, and

screening for colon cancer from the measuring of the Prox-1 expression ~~or activity~~, wherein elevated Prox-1 expression ~~or activity~~ detected in the colon tissue compared to Prox-1 expression in healthy colon tissue correlates with the presence of colon cancer in colon tissue.

2. (Canceled)

3. (Currently amended) A method according to claim [[2]] 1, further comprising a step, prior to said measuring step, of obtaining the biological sample comprising colon tissue from a ~~mammalian~~ the human subject.

4. (Previously presented) The method according to claim 1, wherein the measuring comprises measuring Prox-1 expression in the colon tissue.

5. (Previously presented) The method according to claim 1, wherein the measuring comprises measuring Prox-1 protein in the biological sample.

6. (Original) The method of claim 5, wherein the measuring comprises contacting the colon tissue with a Prox-1 antibody or antigen-binding fragment thereof.

7. (Previously presented) The method of claim 1, wherein the measuring comprises measuring Prox-1 mRNA in the colon tissue.

8. (Original) The method of claim 7, wherein the measuring comprises *in situ* hybridization to measure Prox-1 mRNA in the colon sample.

9. (Original) The method of claim 7, wherein the measuring comprises steps of isolating mRNA from the colon tissue and measuring Prox-1 mRNA in the isolated mRNA.

10. (Previously presented) The method according to claim 1, wherein the measuring comprises quantitative polymerase chain reaction (PCR) to quantify Prox-1 mRNA in the colon tissue relative to Prox-1 mRNA in healthy colon tissue.

11. (Currently amended) A method according to claim 1, further comprising measuring expression ~~or activity~~ of at least one gene selected from the group consisting of cluster of differentiation 44 (CD44), ectodermal-neural cortex protein 1 (Enc1), and inhibitor of DNA binding 2 (ID2) in the colon tissue, and screening for colon cancer from the measuring of the Prox-1 expression ~~or activity~~ and from the measuring of the expression ~~or activity~~ of the at least one gene, wherein elevated Prox-1 expression ~~or activity~~ and elevated expression ~~or activity~~ of the at least one gene in the colon tissue correlate with the presence of colon cancer in colon tissue.

12. (Currently amended) A method according to claim 1, further comprising measuring activation of β -catenin/TCF pathway in the colon tissue, and screening for colon cancer from the measuring of the Prox-1 expression ~~or activity~~ and from the measuring of activation of β -catenin/TCF pathway, wherein activation of the β -catenin/TCF pathway and elevated Prox-1 expression ~~or activity~~ in the colon tissue correlate with the presence of colon cancer in the colon tissue.

13. (Original) A method according to claim 12, wherein activation of the β -catenin/TCF pathway is measured by at least one indicator in the colon tissue selected from the group consisting of: mutations in an APC gene; mutations in a β -catenin gene; and nuclear localization of β -catenin.

14. (Canceled)

15. (Currently amended) A method according to claim ~~[[14]]~~ 1, further comprising a step of administering to a human subject identified as having a colon cancer characterized by increased Prox-1 expression ~~or activity~~ in colon tissue a composition comprising a Prox-1 inhibitor.

16. (Canceled)

17. (Withdrawn/Currently amended) A method of inhibiting the growth of colorectal cancer cells in a ~~mammalian~~ human subject comprising the step of:

administering to the subject a composition comprising a molecule that suppresses expression ~~or activity~~ of Prox-1, thereby inhibiting the growth of colorectal cancer ~~colon~~ ~~carcinoma~~ cells.

18.-20. (Canceled)

21. (Withdrawn) The method according to claim 17, wherein the composition further comprises a pharmaceutically acceptable diluent, adjuvant, or carrier medium.

22. (Withdrawn) The method according to claim 17, wherein the molecule comprises a nucleic acid selected from the group consisting of an antisense oligonucleotide that inhibits Prox-1 expression; micro-RNA that inhibits Prox-1 expression; short interfering RNA (siRNA) that inhibits Prox-1 expression; and short hairpin RNA (shRNA) that inhibits Prox-1 expression.

23.-24. (Canceled)

25. (Withdrawn) The method or use of claim 22, wherein the siRNA comprises at least one nucleotide sequence set forth in SEQ ID NOS: 4, 5, 6, and 7.

26. (Withdrawn) The method of claim 17, wherein the molecule comprises a zinc finger protein that inhibits Prox-1 expression.

27. (Withdrawn) The method of claim 17, wherein the molecule comprises a dominant negative form of Prox-1 protein, or an expression vector containing a nucleotide sequence encoding the dominant negative Prox-1 protein.

28. (Withdrawn) The method of claim 27, wherein the dominant negative form of Prox-1 protein has a disrupted DNA binding domain.

29. (Withdrawn) The method of claim 27, wherein the dominant negative form of Prox-1 protein has a disrupted transactivation domain.

30. (Canceled)

31. (Withdrawn/Currently amended) The method according to claim 17, wherein the composition is administered in an amount effective to suppress Prox-1 expression or activity and increase Notch 1 signaling.

32. (Canceled)

33. (Withdrawn) The method according to claim 17, wherein the composition is administered in an amount effective to increase 15-PDGH activity or decrease prostaglandin D2 synthase activity.

34. (Withdrawn) The method according to claim 17, further comprising administering to the subject an inhibitor of the β -catenin/TCF signaling pathway.

35. (Canceled)

36. (Withdrawn) The method of claim 34, wherein the inhibitor of the β -catenin/TCF signaling pathway is dominant negative form of TCF-4.

37. (Withdrawn) The method of claim 34, wherein the inhibitor of the β -catenin/TCF signaling pathway targets TCF-4, β -catenin, or c-myc.

38. (Withdrawn) The method of claim 17, further comprising administering to the subject a COX-2 inhibitor.

39.-40. (Canceled)

41. (Withdrawn) The method of claim 17, further comprising administering to the subject a Notch signaling pathway agonist.

42.-45. (Canceled)

46. (Withdrawn/Currently amended) A method of inhibiting Prox-1 function in a ~~mammalian~~ human subject having a colon cancer characterized by Prox-1 overexpression in

cells, comprising the step of administering to said ~~mammalian~~ human subject a composition, said composition comprising a compound effective to inhibit Prox-1 function in cells.

47.-67. (Canceled)

68. (Withdrawn) The method of claim 17, wherein the molecule comprises a compound comprising a nucleic acid 8 to 50 nucleotides in length, wherein said compound specifically hybridizes with a polynucleotide encoding Prox-1, or hybridizes to the complement of the polynucleotide, and inhibits the expression of Prox-1 when introduced into a cell that expresses Prox-1.

69. (Canceled)

70. (Withdrawn) The method of claim 22, wherein the antisense oligonucleotide has a sequence complementary to a fragment of SEQ ID NO: 1.

71. (Withdrawn) The method of claim 70, wherein the fragment of SEQ ID NO: 1 comprises a promoter or other control region, an exon, an intron, or an exon-intron boundary.

72. (Withdrawn) The method of claim 70, wherein the fragment of SEQ ID NO: 1 comprises an exon-intron splice junction.

73. (Withdrawn) The method of claim 70, wherein the fragment of SEQ ID NO: 1 comprises a region within 50-200 bases of an exon-intron splice junction.

74. (Withdrawn) The method of claim 17, wherein the molecule comprises an inhibitor of DNA methyltransferases, thereby inhibiting Prox-1 expression.

75. (Withdrawn) The method according to claim 74, wherein the inhibitor of DNA methyltransferases is 5-aza-2'-deoxycytidine.

76. (Withdrawn) The method according to claim 22, further comprising administering to the subject an inhibitor of DNA methyltransferases.

77.-78. (Canceled)

79. (Currently amended) The method according to claim 1, ~~wherein the mammalian subject is human, and~~ wherein the measuring step indicates that the human subject has elevated Prox-1 expression in colon tissue, and the screening step method further comprises diagnosing the human subject as having colon cancer with respect to a cancerous condition of the colon, wherein increased Prox-1 expression or activity in the colon tissue is indicative of a cancerous condition.

80-81. (Canceled)

82. (Withdrawn/Currently amended) A method of selecting patients for therapy with a Prox-1 inhibitor comprising: (a) screening a colon tissue sample ~~cancer~~ from a mammalian human subject for elevated Prox-1 expression compared to the level of Prox-1 expression in a healthy colon tissue sample, wherein elevated Prox-1 expression in the colon tissue sample correlates with the presence of colon cancer cells; and (b) selecting for treatment with a Prox-1 inhibitor a ~~mammalian~~ human subject identified according to (a) as having elevated Prox-1 expression in colon cancer cells.

83. (Canceled)

84. (Withdrawn/Currently amended) A method according to claim 83, further comprising a step, prior to said measuring step, of obtaining a biological sample comprising colon tissue from a ~~mammalian~~ human subject.

85. (Withdrawn/Currently amended) The method of claim 82, further comprising administering to a ~~mammalian~~ human subject identified as having colon cancer with elevated Prox-1 expression a Prox-1 inhibitor selected from the group consisting of: an antisense oligonucleotide that inhibits Prox-1 expression; micro-RNA that inhibits Prox-1 expression; short interfering RNA (siRNA) that inhibits Prox-1 expression; short hairpin RNA (shRNA) that inhibits Prox-1 expression; a zinc finger protein that inhibits Prox-1 expression; a dominant negative form of Prox-1 protein, and an expression vector containing a nucleotide sequence encoding the dominant negative Prox-1 protein.

86. (New) A method of screening colon tissue for colon cancer, said method comprising:

measuring (prospero homeobox protein 1) Prox-1 expression in a biological sample that comprises colon tissue from a human subject, and

screening for colon cancer from the measuring of the Prox-1 expression or activity, wherein Prox-1 expression in the colon tissue in an amount comparable to Prox-1 expression or activity in a colon cancer tissue sample correlates with the presence of colon cancer in colon tissue.

87. (New) The method according to claim 86, wherein the measuring comprises measuring Prox-1 expression in the colon tissue.

88. (New) The method according to claim 86, wherein the measuring comprises measuring Prox-1 protein in the biological sample.

89. (New) The method of claim 88, wherein the measuring comprises contacting the colon tissue with a Prox-1 antibody or antigen-binding fragment thereof.

90. (New) The method of claim 86, wherein the measuring comprises measuring Prox-1 mRNA in the colon tissue.

91. (New) The method of claim 90, wherein the measuring comprises *in situ* hybridization to measure Prox-1 mRNA in the colon sample.

92. (New) The method according to claim 86, wherein the measuring step indicates that the human subject has elevated Prox-1 expression in colon tissue, and the screen step comprises diagnosing the human subject as having colon cancer.

Exhibit B

Expression and prognostic value of the transcription factor Prox1 in colorectal cancer

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Abstract

It has been shown that Prox1 is a specific target of the β -catenin/TCF pathway in the intestinal epithelium and acts as a regulator of progression from a benign to highly dysplastic phenotype in colorectal tumors. However, the clinical significance of Prox1 expression is not known. The objective of this study was to examine the prognostic value of immunohistochemical expression of Prox1 in a series of 517 patients with colorectal cancer. Our results show that the majority of the tumor samples expressed Prox1 (91%, 471/517), whereas normal intestinal epithelium was negative for Prox1, with the exception of a few crypt and neuroendocrine cells. High Prox1 expression was associated with poor grade of tumor differentiation ($p < 0.0001$). In the subgroup of patients with colon cancer, high Prox1 expression was associated with unfavorable colon cancer-specific survival (CCSS) as compared to low Prox1 expression (CCSS 47% vs 59%; $p = 0.04$; RR 1.48). The association between high Prox1 and poor outcome was further strengthened in female colon cancer patients (CCSS 38% vs 61%; $p = 0.007$; RR 2.02). In rectal cancer patients no association between Prox1 and patient outcome was detected (CCSS 49% vs 46%; $p = 0.40$; RR 0.82). In the multivariate survival analysis, adjusted for clinicopathological characteristics, Prox1 expression was not retained as an independent prognostic factor. We conclude that high Prox1 expression is associated with histologic grade of differentiation and in the subgroup of colon cancer patients also with less favorable patient outcome. Our results from this patient series strengthen the previous preclinical observations that Prox1 plays a role in tumor progression.

Key words

Prospero-related homeobox gene, Prox1, colorectal cancer, expression, prognosis, survival analysis

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world (1). Up to 15% of CRCs occur in dominantly inherited patterns. The two best defined familial forms are Familial Adenomatous Polyposis (FAP) related colorectal cancer and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (2). Activation of the APC/ β -catenin/TCF pathway is an initiating event in neoplasia in FAP patients. The *adenomatous polyposis coli* (*APC*) and *β -catenin* (*CTNNB1*) genes are two major components of the Wnt signaling pathway that are affected by mutations in CRC (3). In normal cells the APC protein binds cytoplasmic β -catenin targeting it for degradation. When the degradation is inhibited by Wnt signaling, β -catenin begins to accumulate in colorectal epithelial cell nuclei. Wnt signaling results in formation of a complex containing β -catenin and T-cell factor (TCF). FAP patients with *APC* mutation and blocked β -catenin degradation have an overactivated Wnt signaling pathway, which results in development of hundreds of intestinal polyps, and eventually colorectal cancer.

Loss of *APC* in the intestinal epithelium induces expansion of the progenitor cell population. The β -catenin/TCF pathway controls cancer cell proliferation and expression of progenitor cell-specific genes (4). In humans, progression from benign adenoma to malignant carcinoma takes several years and the cascade causing this malignant transformation still remains unclear. Petrova et al. (2008) recently showed that the transcription factor Prox1 is an intestinal specific target of the β -catenin/TCF pathway and has an essential role as a regulator of progression from a benign to highly dysplastic phenotype in colorectal tumors.

Prox1 is an atypical homeodomain protein important for embryonic development of the lens, retina, liver, pancreas, and lymphatic vasculature (6, 7, 8, 9), but little is known about Prox1 function in adult tissues. Prox1 is the mammalian homolog of the *Drosophila* homeobox protein *Prospero*, which acts as a brain tumor suppressor by inhibiting neuroblast self-renewal (7, 10). It has been suggested that Prox1 has a similar role in human cancer (11, 12). In

contrast, Petrova et al. (2008) showed that Prox1 is overexpressed in the majority of CRCs and promotes neoplasia, tumor growth, and malignant progression. These findings suggest that expression of Prox1 might be associated with outcome of CRC patients, but the clinical significance of Prox1 is not known. In the present study we investigated the expression of Prox1 in a large series of colorectal cancer patients.

Patients and methods

Patients

The study is based on a series of 643 consecutive patients who underwent surgery for histologically verified colorectal cancer at the Helsinki University Central Hospital in 1989 to 1998. The median follow-up time of the patients alive at the end of follow-up was 9.0 years (range 0.1 to 15.4). A tissue specimen suitable for evaluation of Prox1 expression by immunohistochemistry was available in 517 (80.4%) cases. Follow-up data were available for all patients and was collected from the patient records, and the files of the Finnish Cancer Registry and Statistics Finland. The clinicopathological characteristics of the patients in this series have been described in detail previously (13; Table 1).

Immunohistochemistry

Prox1 expression was assessed from tissue microarrays prepared as described elsewhere (13). Three 1.0 mm cores were biopsied from each paraffin-embedded tumorblock. The tissue microarray blocks were cut into 4 µm thick sections, fixed on slides and dried for 12 to 24 hours at 37°C. The sections were deparaffinized in xylene and rehydrated through graded alcohol series. For antigen retrieval, the sections were heated in the Pretreatment Module of the Autostainer 480 (LabVision UK Ltd, Newmarket, UK) in Tris-EDTA buffer (pH 9.0) for 20 min at 98°C. Staining of sections was performed in Autostainer 480. The tissue sections were treated with 0.3% Dako REAL Peroxidase-Blocking Solution (Dako Denmark A/S, Glostrup, Denmark) for 30 min to block endogenous peroxidases, followed by incubation with rabbit normal serum (Vectastain ABC Kit, Vector, Burlingame, CA, USA) diluted 1:50 in TNB blocking solution [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Blocking reagent (supplied in kit); Renaissance®

TSA™ Biotin System; PerkinElmer™, Boston, MA, USA] for 30 min. Goat anti-Prox1 antibody (R&D Systems, Minneapolis, MN, USA) was used to detect Prox1 expression. The antibody was diluted 1:2000 in TNB blocking solution and incubated with the samples overnight at +4°C. After overnight incubation with primary antibody, the tissue sections were incubated for one hour with a biotinylated anti-goat secondary antibody (Vectastain ABC Kit), diluted 1:300 in TNB blocking solution, and treated for 30 min with Streptavidin-HRP Conjugate (PerkinElmer™) diluted 1:1250 in TNB blocking reagent. Immunostaining was visualized with Dako REAL Diaminobenzidine (DAB) Chromogen (10 min treatment). Between each step in the staining procedure, the slides were washed with wash buffer (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, 0.04% Tween®20; pH 7.4). Finally, the slides were counterstained with Meyer's hematoxylin, washed in tap water for 10 min, and mounted in aqueous mounting medium (Aquamount, BDH, Poole, UK). Specificity of the Prox1 immunopositivity was confirmed by staining tissue without the primary antibody.

Scoring of Prox1 immunostaining

Expression of Prox1 was evaluated by two of the investigators (L.C.A. and M.S.). Both investigators were blinded to the clinicopathological data at the time of scoring. Prox1 staining in the cancer cell nuclei was scored as follows: 0 = negative, no staining in cancer cells; 1 = low, less than 25% of cancer cells stained positively for Prox1, intensity of staining was weak; 2 = moderate, 25-50% of cancer cells were positive for Prox1; 3 = strong, 50-75% of cancer cells positive; 4 = very strong, more than 75% of cancer cells positive. If more than one tissue spot was available for the same patient, the highest score out of the parallel spots was selected for statistical analysis.

Statistical analyses

The association between Prox1 immunohistochemistry results and clinicopathological variables was assessed by using the χ^2 test. Life tables were computed according to the Kaplan-Meier method. Colorectal cancer-specific survival (CCSS) was calculated from the date of the diagnosis to death from colorectal cancer. Patients who died from causes other than colorectal cancer were censored on the date of death. Survival between groups was compared using the logrank test. Multivariate survival analyses were carried out using the Cox proportional hazards model and a p-value of 0.05 was adopted as the limit for inclusion of a covariate. All p-values are 2-tailed.

Results

Prox1 expression in colorectal cancer

Prox1 expression was localized in tumor cell nuclei. Normal epithelium was mostly negative for Prox1, but expression could be observed in a few crypt and neuroendocrine cells and in the nuclei of lymphatic vessel endothelium. Prox1 immunoreactivity was absent in only 9% (46/517) of all investigated colorectal cancers. The majority of the tumor samples showed some degree of Prox1 expression (91%, 471/517). Low Prox1 expression was detected in 24% (122/517) of the tumors, moderate in 43% (224/517), strong in 20% (105/517), and very strong expression in 4% (20/517) of the tumors. Prox1 immunoreactivity was absent in 9% (46/517) of all investigated colorectal cancers. Representative immunostaining results are shown in Figure 1.

Association between Prox1 expression and clinicopathological parameters

High Prox1 expression was significantly more frequent in high grade tumors (grade 3-4) compared to low grade (grade 1-2) tumors ($p=0.0001$; Table 1). None of grade 1 tumors had high Prox1 expression. No statistically significant association was found between Prox1 and age at diagnosis, tumor location, tumor site, gender or Dukes stage (Table 1). Only 7% of well-differentiated tumors were Prox1 negative whereas 13% of poorly differentiated tumors were negative for Prox1 (Table 2).

Association of Prox1 expression with colorectal cancer-specific survival

For further analyses we categorized the patients into two groups, Prox1 low (staining scores from 0 to 2) and Prox1 high (scores 3 and 4). High Prox1 expression was not significantly asso-

ciated with CCSS of colorectal cancer patients (RR=1.14; p=0.38; Table 3, Figure 2A). The 5-year CCSS was 57% [95% confidence interval (CI), 52.1-62.5%] among patients with low Prox1 expression level, and 53% (95% CI, 43.6-62.1%) when the Prox1 expression was high.

In subgroup analyses, high Prox1 expression was associated with unfavorable survival in colon cancer patients (RR=1.48; p=0.04; Table 3, Figure 2B). The 5-year colorectal cancer specific survival of the colon cancer patients with low Prox1 expression was 62% (95% CI, 55.2-68.9%) as compared to 47% for those with high staining intensity (95% CI, 35.3-59.0%). The 5-year CCSS among colon cancer patients with very high (score 4; n=11) Prox1 expression was only 24% suggesting that increasing expression of tumor cell Prox1 is associated with worse outcome of the colon cancer patients. Furthermore, the 5-year CCSS of female colon cancer patients with high Prox1 expression (score 3-4) was 38% (95% CI, 22.0-54.7%) compared to 63% when the expression of Prox1 was low (95% CI, 53.3-73.0%; p=0.007; Figure 2C), whereas no difference was detected among male colon cancer patients. No significant association was detected between Prox1 and survival in rectal cancer patients since 5-year CCSS for Prox1 low patients was 51% (95% CI, 43.6-59.4%) and 61% for Prox1 high patients (95% CI, 47.1-75.9%; p=0.4; Table 3, Figure 2D).

Multivariate survival analysis

To adjust for established prognostic factors in colorectal cancer, Prox1 expression was entered into a Cox proportional hazards model together with Dukes stage, histologic grade, age at diagnosis, tumor location, site, and gender. In the multivariate survival analysis Prox1 expression was not a significant prognostic factor (Table 4). Cox multivariate analysis was also performed for the subgroup of female colon cancer patients, however, Prox1 expression did not add significant prognostic information to that of Dukes stage, histologic grade, tumor location and age at diagnosis (data not shown).

Discussion

To explore the clinical significance of Prox1, we investigated expression of Prox1 by immunohistochemistry in tissue microarray specimens of 517 patients with colorectal cancer. The present results indicate that high Prox1 expression is associated with the grade of tumor differentiation and with less favorable prognosis in the subgroup of patients with colon cancer. Moreover, our data shows that high Prox1 expression is associated with unfavorable outcome in the subset of female colon cancer patients. These observations are in line with the hypothesis that Prox1 overexpression promotes the progression of colon cancer (5).

We found nuclear Prox1 expression in 91% of the 517 colorectal cancer specimens, and 27% of these samples showed high level of expression. The current study showed that high Prox1 expression was associated with high tumor grade, but not with other clinicopathological parameters. Currently tumor grade is an important clinical indicator of prognosis in colorectal cancer. Distinct differentiation status may indicate different biological behavior of the cancer. In the present study, none of the grade 1 tumors had a high Prox1 expression. The reason for the lack of Prox1 expression in highly differentiated tumors remains unknown, but this could be due to, e.g., mutation or epigenetic alteration of the gene, but more studies are needed to address the mechanism of Prox1 action.

The current study shows that Prox1 has prognostic value among colon cancer patients, whereas no difference was found in rectal cancer patients (Table 2, Figure 2). A common assumption is that rectal carcinomas arise through similar mechanisms as left-sided colon tumors and have thus been included in this category. It has been suggested that prognosis for right-sided colon cancers is different from left-sided colon cancers (14). Various proposals have been given to explain this hypothesis, including environmental factors, genetic factors and sex distribution (15). During embryonic development, the right colon arises from the midgut and the left colon from the hindgut. Analysis of genetic databases from normal colon and tumor speci-

mens has revealed differences in gene expression between normal mucosas and between colon carcinomas originating from the right and left colons (16, 17). It was recently shown in a large population-based study that right-sided colon cancers have a worse prognosis than left-sided cancers and that women are more likely to get right-sided colon cancer, although women did not have significant difference in mortality between left- and right-sided colon cancer (18). In the present study, the proportion of right-sided versus left-sided tumors was similar in males and females and we found no significant difference in Prox1 expression between right-sided and left-sided tumors (data not shown). Since only few studies have addressed the possible biological differences between colon and rectal cancer, further studies are necessary to clarify the molecular differences (Kapiteijn et al., 2001).

As compared to other cancer types, very few molecular prognostic markers have been reported in colon cancer. Molecular markers for tumor tissue would be important for making clinical decisions, since targeted therapy for each patient is an important goal for improving the outcome of patients with CRC. To date, the exact mechanism of Prox1 action in normal and diseased tissue is poorly understood. Thus, further studies regarding the molecular mechanisms that regulate Prox1 expression and the direct target genes of Prox1 are needed. In summary, our results show that high nuclear Prox1 expression is associated with unfavorable outcome in colon cancer patients and, moreover, in the subset of female colon cancer patients. In addition, these results confirm the previous preclinical observations that Prox1 plays a role in tumor progression.

Acknowledgments

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Figure legends

Figure 1. Prox1 expression in human colorectal cancer tissue microarray specimens. *A*, Low Prox1 expression; only few cancer cell nucleus are positive. *B*, moderate Prox1 expression. *C*, strong Prox1 expression. *D-E*, very strong Prox1 expression. *F*, negative for Prox1. Magnification 200x *D*, 400x *A-C*, *E-F*.

Figure 2. Disease-specific survival of 517 colorectal cancer patients according to Prox1 expression. *A*, all colorectal cancer patients. “Prox1 low”, scores from 0 to 2, n=392; “Prox1 high”, scores 3 and 4, n=125. *B*, colon cancer patients. “Prox1 low”, n=218; “Prox1 high”, n=73. *C*, female colon cancer patients. “Prox1 low”, n=101; “Prox1 high”, n=36. Rectal cancer patients. “Prox1 low”, n=174; “Prox1 high”, n=52.

Table 1. Associations of Prox1 expression with clinicopathological variables.

Table 2. Association of Prox1 expression with histologic grade.

Table 3. Five-year cancer-specific survival (CCSS) of 516 patients with colorectal cancer according to nuclear Prox1 expression.

Table 4. Cox multivariate regression of the association between Prox1 immunoreactivity and colorectal cancer-specific survival, adjusted for clinicopathological characteristics (n=516).